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BIOLOGY

Activation and Expansion of CD8$^+$ T Effector Cells in Patients with Chronic Graft-versus-Host Disease

Bryan M. Grogan,[1] Laura Tabellini,[1] Barry Storer,[1,2] Tara E. Bumgarner,[1] Claudia C. Astigarraga,[1] Mary E. D. Flowers,[1,2] Stephanie J. Lee,[1,2] Paul J. Martin,[1,2] Edus H. Warren,[1,2] John A. Hansen[1,2]

We tested the hypothesis that changes in the phenotype of CD8$^+$ T cells from patients with chronic graft-versus-host disease (cGVHD) correlate with disease activity, and resolve or normalize in clinically tolerant patients successfully withdrawn from immunosuppression therapy (IST). No significant difference was found in the absolute CD8$^+$ T cell counts among cGVHD patients, tolerant patients, and healthy controls. However, compared with healthy normal controls, CD8$^+$ T cells from cGVHD patients had decreased expression of the IL-7 receptor and an increase in effector T cells, Ki-67, and perforin expression and apoptosis, suggesting that activation, differentiation, and proliferation of host-reactive CD8$^+$ effector T cells is a mechanism by which cGVHD is sustained and persists. The increase in effector T cells was most prominent in older patients and patients who were cytomegalovirus seropositive before transplantation. Use of IST was associated with a decreased number of CD45RA$^-$ CD8$^+$ effector T cells, a decreased expression of Ki-67, and an increased expression of CD95 (Fas). Together, these results demonstrate that CD8$^+$ T cells in patients with cGVHD are characterized by an increased level of activation and proliferation, and an expansion of effector cells that appear to be selectively sensitive to IST compared with other CD8$^+$ T cells. In GVHD-free tolerant patients, CD8$^+$ T cells showed an increased expression of granzyme and HLA-DR molecules compared with CD8$^+$ T cells from healthy controls, indicating that clinical tolerance in these patients can occur without full normalization of the CD8$^+$ T cell phenotype.

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INTRODUCTION

Chronic graft-versus-host disease (cGVHD) is a frequent complication of allogeneic hematopoietic cell transplantation (HCT), affecting 40% to 60% of patients with onset occurring within 6 to 24 months post-HCT [1]. Chronic GVHD is more likely to occur in patients who have previously had acute GVHD (aGVHD), but not all patients with resolved aGVHD develop cGVHD. Chronic GVHD can also develop in the absence of prior aGVHD [2-4]. Duration of cGVHD is variable and on average requires systemic immunosuppression therapy (IST) for a median of 24 to 36 months [5]. Absence of aGVHD or cGVHD activity after withdrawal of IST has been interpreted as indicating the development of clinical immunologic tolerance [1,2,5,6]. It is unknown, however, whether "clinical tolerance" following HCT meets the classical experimental definition of immunologic tolerance. The mechanism by which cGVHD evolves into quiescence characterized by lack of clinical signs and symptoms without need for immune suppressive medications is also unknown.

Donor T cells are recognized as the primary mediators of aGVHD, but less is known about the role of T cells in cGVHD. It is commonly assumed that cGVHD is mediated and sustained by donor T cells reactive to host-specific alloantigen, although immune dysfunction and autoreactivity may also contribute to the pathologic lesions characteristic of cGVHD [7,8]. Two previous studies have examined CD8$^+$ T cells in patients with cGVHD. Yamashita et al. [9] reported a significant increase in CD45RA$^-$CCR7$^+$ central memory T cells, and D'Asaro et al. [10] showed an increase in CD45RA$^+$CCR7$^-$ effector T cells in patients with cGVHD. In the study reported here, we performed a cross-sectional study of patients returning at various times posttransplantation for

From the [1]Fred Hutchinson Cancer Research Center, Seattle, Washington; and [2]University of Washington, Seattle, Washington.
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Correspondence and reprint requests: John A. Hansen, MD, Human Immunogenetics Program, Fred Hutchinson Cancer Research Center, Seattle, WA 98087 (e-mail: jhansen@fhcrc.org).
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assessment and management of cGVHD. Research blood samples were obtained and examined for the relative numbers of naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and effector (T_{EFF}) $CD8^+$ T cell subsets as well as T cell effector molecules and activation markers to test the hypotheses (1) that changes in $CD8^+$ T cell phenotype in cGVHD patients correlates with disease activity, (2) that these changes resolve or normalize in clinically tolerant patients who have been successfully withdrawn from IST without recurrence of GVHD, and (3) that IST use is associated with specific CD8 phenotypes in patients with cGVHD. Our major findings include an increase in $CD8^+$ T_{EFF} cells overall, an increased expression of perforin and Ki-67, and a decrease in T_N cells in patients with cGVHD, along with a decrease in $CD45RA^-$ T_{EFF} cells during IST. T_{EFF} levels, and perforin and Ki-67 expression were normal in tolerant patients; however, tolerant patients also showed an unexpected increase in the expression of HLA-DR and granzyme A. These results overall provide a framework for future functional studies aimed at defining the mechanism(s) by which clinical tolerance occurs following HCT.

METHODS

Study Population and Samples

The study population included 273 patients who had a comprehensive history and physical examination for the presence or absence of cGVHD at our Long-Term Follow-Up Clinic. All patients received T cell replete bone marrow (n = 53), mobilized peripheral blood cells (n = 218), or umbilical cord blood (n = 2), from a related (n = 146) or unrelated (n = 127) donor, for the treatment of a hematologic malignancy or myelodysplasia. Cyclosporine or tacrolimus, and short methotrexate or mycophenolate mofetil, were given for aGVHD prophylaxis. Heparinized blood was obtained from patients at a median of 28 months post-HCT (range: 6-271 months), and from healthy normal volunteers who had no history of HCT ("controls"). The median age of the patients at the time of transplantation was 46 years (range: 12-73), and the median age of their donors was 38 years (range: 0-76). The median age of healthy normal controls was 37 years (range: 22-73). Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density centrifugation, washed, and aliquoted for cryopreservation in 10% DMSO and 90% fetal bovine serum, and were later thawed for cell surface and intracellular staining. All participants gave informed consent according to institutional review board approved protocols.

Classification of cGVHD

Graft-versus-host disease (GVHD) status was evaluated at the time that blood samples were obtained. A diagnosis of clinical cGVHD was established according to the recommendations of the National Institutes of Health Consensus Conference [11,12]. Clinical tolerance was defined as the absence of all reversible signs and symptoms of GVHD in patients withdrawn from all IST for at least 3 months at the time of blood collection and with 6 months of additional follow-up without recurrence of cGVHD. Patients were classified for evidence of disease activity and use of IST and assigned to 1 of 4 categories as

Systemic Immunosuppressive Treatment	GVHD Activity [1]: Absent	GVHD Activity [1]: Present
No	**A)** Discontinued prophylaxis without prior chronic GVHD, or discontinued immunosuppressive treatment after complete resolution of all reversible manifestations of chronic GVHD	**B)** Manifestations of mild chronic GVHD (NIH criteria) without need for systemic immunosuppressive treatment, or manifestations of moderate or severe chronic GVHD (NIH criteria) before onset of systemic immunosuppressive treatment
Yes	**C)** Systemic immunosuppressive treatment without manifestations of chronic GVHD	**D)** Manifestations of classic chronic GVHD or overlap syndrome (NIH criteria), and systemic immunosuppressive treatment

[1] Chronic GVHD activity is assessed by clinical examination and routine laboratory studies according to NIH criteria.

Figure 1. Classification of GVHD by clinical activity and use of immunosuppression therapy (IST). The patients included in this study were classified into 1 of 3 groups: (A) tolerant patients, with absence of any clinical signs of GVHD and not currently on any form of systemic IST; (B) chronic GVHD patients without IST; and (D) chronic GVHD patients receiving IST. Patients with "controlled" or quiescent GVHD (C) who were still receiving IST were excluded from the study.

illustrated in Figure 1. Patients with "controlled" or quiescent cGVHD who were still on IST were excluded from the study. The remaining patients were classified into 3 groups as follows: (1) cGVHD, receiving IST; (2) cGVHD without IST; and (3) tolerant, either with no history of cGVHD or after recovery from cGVHD.

Cell Surface and Annexin Staining

Thawed peripheral blood mononuclear cells were examined by flow cytometry after staining with a series of fluorochrome-labeled antihuman monoclonal antibodies for cell-surface molecules. Cells subjected to 3-color staining were analyzed on a BD FACScan and cells stained for >3 colors were analyzed on a BD LSRII. The collected data were further analyzed with the use of BD CellQuest Pro software. Reagents included CD8 FITC, CD95 (FAS) FITC, CD127 (IL-7Rα) PE, CD8 PE-Cy5, HLA-DR PerCP-Cy5.5, CD279 (PD-1) APC, and CD3 APC-Cy7 (BD Pharmingen, San Diego, CA), CD62L PerCP-Cy5.5, CD45RA Pacific Blue, and CD62L Alexa Fluor 700 (Biolegend, San Deigo, CA). Isotype-matched negative control antibodies included IgG1κ FITC, IgG1κ PE, IgG2aκ PerCP-Cy5.5, IgG1κ APC, IgG1κ APC-Cy7, and IgG1κ Alexa Fluor 700 isotype antibodies (BD Pharmingen), and with IgG1κ PerCP-Cy5.5, IgG1κ PE-Cy7, IgG2bκ APC, and IgG2bκ Pacific Blue (Biolegend). Apoptosis was assessed by staining thawed cells with Annexin V FITC (Apoptosis Detection Kit I, BD Pharmingen) at 24 hours after in vitro culture in RPMI 1640 (Gibco, Gaithersburg, MD) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in a CO_2 incubator at 37°C.

Intracellular Staining

Staining of $CD8^+$ T cell subsets for cytoplasmic proteins was accomplished in a 2-step process. First, cell-surface staining was performed, then cells were fixed and permeabilized for intracellular staining with the BD Pharmingen Cytofix/Cytoperm fixation/permeabilization reagents and protocol according to the manufacturer's instructions, and stained with the following antibodies: FITC-conjugated Ki-67 (BD Pharmingen); PerCP-Cy5.5-conjugated Granzyme A and APC-conjugated Perforin (Biolegend).

Definition of $CD8^+$ T Cells

Two populations of $CD8^+$ T cells can be identified according to the expression level of CD8 as $CD8^{bright}$ and $CD8^{dim}$. Approximately 80% of the $CD8^+$ population stained $CD8^{bright}$ in both patients and normal controls (data not shown). The composition of the $CD8^{dim}$ and $CD8^{bright}$ subsets were examined in detail in 46 patients and 9 controls by staining for CD3, CD56, and CD4. The majority of the $CD8^{dim}$ cells in both patients and controls were $CD56^+$ (62.0% ± 2.5% and 64.8% ± 3.3%, $P = .50$), and <25% were $CD3^+CD4^-CD56^-$ (22.5% ± 1.7% and 23.8% ± 4.2%, respectively), indicating that the $CD8^{dim}$ population consisted mostly of natural killer or natural killer T cells. In contrast, 87.4% ± 1.1% of patient and 88.3% ± 2.2% of control $CD8^{bright}$ cells were $CD3^+CD4^-CD56^-$. Based on these findings, we restricted our study to the $CD8^{bright}$ lymphocyte population, thus excluding from the subsequent analysis natural killer and natural killer T cells [13,14].

Definition of $CD8^+$ T Cell Subsets

CD8 subsets were characterized according to expression of CD62L and CD127 as previously described by Bachmann et al. [19] and Huster et al. [20]. Staining with CD45RA was included to help distinguish naïve and central memory $CD8^+$ T cells, and to serve as a marker for identifying the $CD45RA^-$ and $CD45RA^+$ $CD8^+$ T_{EFF} subsets. $CD8^+$ T cells were gated to define subsets in the following fashion: naïve (T_N), $CD62L^+CD127^+CD45RA^+$; central memory (T_{CM}), $CD62L^+CD127^+CD45RA^-$; and effector memory (T_{EM}), $CD62L^-CD127^+CD45RA^-$. $CD8^+$ effector (T_{EFF}) T cells were defined as $CD62L^-$ $CD127^-$. CD45RA expression was used to further subdivide T_{EFF} into RA^+ and RA^- subsets (illustrated in Figure 2).

Statistical Analysis

Multivariate regression analysis was used to compare T cell counts among patients who had cGVHD without IST, cGVHD during IST, tolerant patients, and healthy normal controls, adjusting for age of patients and controls. Clinical variables that might affect the observed T cell counts and phenotype including hematopoietic cell source (marrow, peripheral blood), donor relationship (related, unrelated), age of patient at the time of HCT and at the time of study, cytomegalovirus (CMV) serostatus of the patient before HCT, sex mismatch (female donor for a male recipient, other), cGVHD status at the time of study (presence, absence), use of IST (yes, no), history of aGVHD (yes, no), history of cGVHD (yes, no), and time from transplantation were examined in a multivariate regression model (results of the multivariate analysis are summarized in Supplemental Tables S1-S3). P values were calculated using a 2-tailed t test and were adjusted for age at the time of sample collection when comparing patients and normal controls. Donor age was also examined but had no significant effect on the numbers or phenotypes of T cells after HCT (data not shown). Pearson correlation coefficients were calculated to evaluate the relationship between age and T cell counts in patients and controls. All P values are 2 sided and are not adjusted for multiple

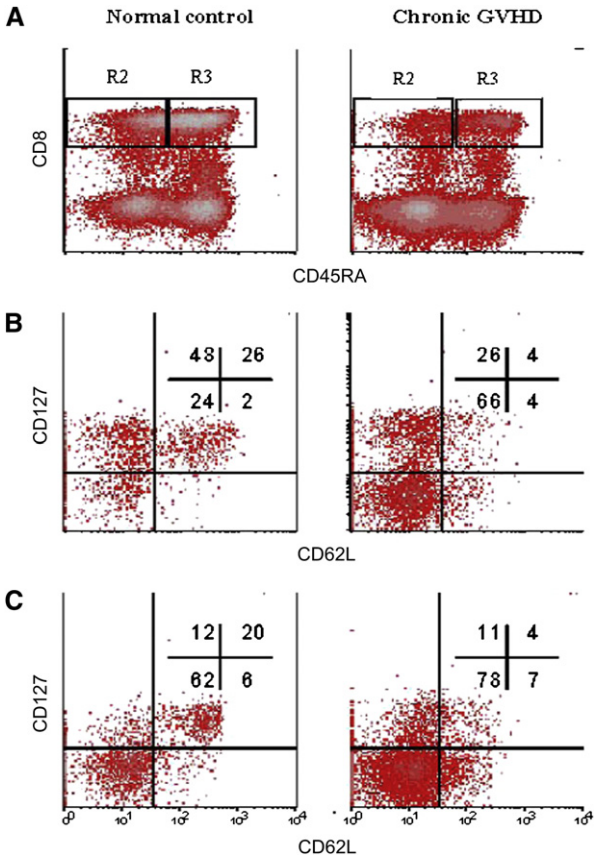

Figure 2. Staining of CD8 subsets. Thawed peripheral blood mononuclear cells were stained with CD8−FITC, CD45RA−Pacific Blue, CD127-PE, and CD62L-PerCP-Cy5.5 antibodies and PBL were gated using CellQuest Pro. (A) Subsequent gating was performed on $CD8^{bright}CD45RA^{-}$ (gate R2) and $CD8^{bright}CD45RA^{+}$ (gate R3) cells. (B) The $CD45RA^{-}$ population (gate R2) was further divided into quadrants based on CD127 and CD62L expression to define the following populations: $CD127^{+}$ $CD62L^{-}$ T_{EM} (UL), $CD127^{+}$ $CD62L^{+}$ T_{CM} (UR), and $CD127^{-}$ $CD62L^{-}$ RA^{-} T_{EFF} (LL). (C) The $CD8^{+}CD45RA^{+}$ population (gate R3) was divided in a similar fashion to define the $CD127^{+}CD62L^{+}$ T_{N} (UR) and $CD127^{-}$ $CD62L^{-}$ $RA^{+}T_{EFF}$ (LL) populations. The same healthy control and chronic GVHD patients, who were off IST, were used for illustration in each panel. Percentages displayed represent percent of $CD8^{bright}$ $CD45RA^{-}$ (R2) or percent of $CD8^{bright}$ $CD45RA^{+}$ (R3).

comparisons. P values $\leq .01$ were considered statistically significant, whereas P values between $>.01$ and $\leq .05$ were considered marginally significant.

RESULTS

T Cell Counts in cGVHD and Tolerant Patients

The absolute number of $CD3^{+}$ and $CD8^{+}$ T cells were comparable in cGVHD patients, tolerant patients, and normal controls, but the absolute number of $CD4^{+}$ T cells was significantly decreased in patients with cGVHD (660 ± 35 cells/μL; $P = .009$), especially during IST (635 ± 44 cells/μL, $P = .006$) compared with controls (856 ± 47 cells/μL) (Table 1). The absolute $CD3^{+}$ and $CD4^{+}$ T cell counts were greater with increasing time post-HCT, and higher absolute $CD3^{+}$, $CD4^{+}$, and $CD8^{+}$ T cell counts were observed in CMV-seropositive patients ($P < .001$, $P = .03$, and $P < .001$, respectively). We found no statistically significant effect of patient or donor age, donor type, or use of IST on the numbers of $CD3^{+}$ or $CD8^{+}$ T cells in patients with cGVHD or in tolerant patients (Table S1).

Expression of CD127

CD127, the IL-7 receptor, is essential for the survival of memory T cells, and its expression can be used to define T cell subsets with distinct functional properties [17]. We observed a significant decrease in the expression of CD127 on $CD8^{+}$ T cells from patients with cGVHD compared with normal controls (48% ± 3% versus 72% ± 3%, $P < .001$) (Figure 3). CD127 expression tended to normalize in tolerant patients compared with normal controls (62% ± 4% versus 72% ± 3%, P = ns). In the multivariate regression model, the percentage of $CD8^{+}$ T cells expressing CD127 was lower in older patients (−0.7%/year; $P < .001$) and patients who were CMV seropositive before HCT (−13.5%, $P = .01$) (Table S2A), but was not associated with donor age (data not shown).

Analysis of $CD8^{+}$ T Cell Subsets

The percentage of $CD8^{+}$ T_{EFF} cells was significantly increased in patients with cGVHD overall compared with normal controls (39% ± 3% versus 19% ± 3%, $P < .001$) (Table 2A). There was also a 3-fold increase in the absolute number of T_{EFF} in cGVHD patients overall compared with normal controls (158 ± 32 versus 54 ± 14 cells/μL); however, this difference was only of marginal significance when adjusted for age ($P = .05$) (Table 2B). IST had no significant effect on either the percentage (38% ± 4% during IST versus 40% ± 4% without IST, P = ns) (Table 2A) or the absolute number of T_{EFF} in GVHD patients (144 ± 50 cells/μL during IST versus 171 ± 41 cells/μL without IST, P = ns) (Table 2B). The observed increase in T_{EFF} in patients with cGVHD was balanced by a significant decrease in the percentage and absolute number of T_{N} compared with controls (20% ± 2% versus 35% ± 4%, $P = .001$; and 34 ± 5 cells/μL versus 84 ± 25 cells/μL, $P = .003$, respectively). Although there was a significant decrease in the percentage of T_{EM} in cGVHD patients overall compared with controls (13% ± 1% versus 19% ± 2%, $P = .004$), there was no significant difference in the absolute number of T_{EM} in cGVHD patients overall compared with controls (43 ± 9 cells/μL versus 46 ± 11 cells/μL, P = ns). There was also no significant difference in the percentage or absolute number of T_{CM} cells in cGVHD patients compared with controls (Table 2A-B).

In tolerant patients, neither the percentage nor the absolute number of T_{EFF} were significantly different from normal controls (27% ± 4% versus 19% ± 3%,

Table 1. CD3$^+$, CD4$^+$, and CD8$^+$ T cells in Chronic GVHD and Tolerant Patients*

	Chronic GVHD				
CD3$^+$ T Cells	Without IST (n = 51)‡	During IST (n = 118)	Overall (n = 169)	Tolerant (n = 44)	Normal Controls (n = 33)
abs CD3†	1265 ± 85	1150 ± 77	1185 ± 60	1393 ± 110	1346 ± 69
abs CD4	716 ± 56	**635 ± 44** (0.006)	**660 ± 35** (0.009)	836 ± 76	856 ± 47
abs CD8	468 ± 47	450 ± 40	455 ± 31	441 ± 42	410 ± 36
%CD4	**58 ± 2** (0.05)	**58 ± 2** (0.03)	**58 ± 1** (0.03)	60 ± 2	64 ± 2
%CD8	35 ± 2	**36 ± 1** (0.02)	**36 ± 1** (0.002)	31 ± 2	30 ± 2
CD4:CD8 ratio	2.3 ± 0.3	2.2 ± 0.1	2.2 ± 0.1	2.5 ± 0.3	2.6 ± 0.3

GVHD indicates graft-versus-host disease; IST, immunosuppressive therapy.

*Data consist of mean ± SEM and *P* values. All *P* values represent comparisons between patients and normal controls and are adjusted for age of patients and normal controls. Adjusted *P* values >.05 are omitted.

†"abs" indicates absolute number of cells/μL, and "%" indicates percentage of total CD3$^+$ T cells.

‡The chronic GVHD without IST group includes 41 patients with "mild" and 10 patients with "moderate/severe" disease. No significant differences were detected in the percentages of absolute numbers of CD3, CD4, or CD8 between these two subgroups (data not shown).

P = ns; and 54 ± 12 cells/μL versus 54 ± 14 cells/μL, *P* = ns, respectively). There was also no significant difference in the absolute number of T_N in tolerant patient compared with controls (63 ± 16 versus 84 ± 25 cells/μL, *P* = ns), but there was a trend of marginal significance for a decrease in the percentage of T_N in tolerant patients compared with controls (25 ± 4% versus 35% ± 4%, *P* = .05). There was no significant difference in the percentage and absolute number of T_{CM} and T_{EM} in tolerant patients compared with normal controls (Table 2A-B and Figure 4A-B).

Taken together, the changes in the percentage and absolute numbers of T_{EFF} and T_N demonstrate a marked increase of T_{EFF} in cGVHD patients coupled with a decline in both the percentage and absolute number of T_N, and a decline in the relative percentage of T_{EM}, whereas no significant changes were seen in the percentage or absolute number of T_{CM} cells. These results overall suggest an expansion of T_{EFF} cells by proliferation and/or increased survival, and a decrease in the production or an increase in the recruitment and activation of T_N in cGVHD patients, changes that tended to normalize in tolerant patients.

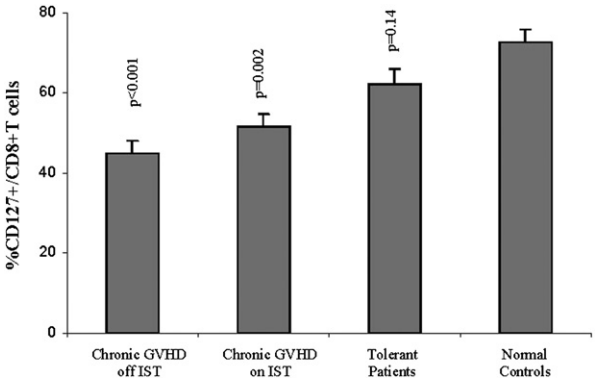

Figure 3. Comparison of CD127 expression on CD8$^+$ T cells in patients with chronic GVHD, tolerant patients, and normal controls. The data are expressed as %CD127$^+$/CD8$^+$ T cells. Samples include 24 patients with chronic GVHD off IST (19 mild and 5 moderate/severe disease), 20 patients with chronic GVHD on IST, 21 tolerant patients, and 20 normal healthy controls. All *P* values represent comparisons between patients and normal controls and are adjusted for age of patients and normal controls.

Additional changes were seen in the T_{EFF} subsets in cGVHD patients, especially during IST. The percentage and absolute number of RA$^-$ T_{EFF} cells were increased in cGVHD patients overall compared with normal controls (15% ± 2% versus 7% ± 1%, *P* = .006; and 67 ± 15 versus 18 ± 5 cells/μL, *P* = .05). This increase was especially prominent in cGVHD patients without IST (20% ± 2%, *P* < .001; and 88 ± 23 cells/μL, *P* = .01). The percentage of RA$^-$ T_{EFF} cells was lower in cGVHD patients during IST (10% ± 2% during IST versus 20% ± 2% without IST, *P* = .009), and there was a lower absolute number of RA$^-$ T_{EFF} cells (43 ± 17 cells/μL during IST versus 88 ± 23 cells/μL without IST), but this apparent difference was not statistically significant. The percentage and absolute number of RA$^-$ T_{EFF} cells in patients during IST were not significantly different from controls (Table 2A-B and Figure 4A-B). The percentage of RA$^+$ T_{EFF} was increased in cGVHD patients overall compared with controls (24% ± 2% versus 12% ± 2%, *P* = .002) together with a trend for an increase in the absolute number of RA$^+$ T_{EFF} cells (92 ± 19 versus 37 ± 10 cells/μL, *P* = .07). These increases in percentage and absolute number of RA$^+$ T_{EFF} were more prominent in cGVHD patients during IST (28% ± 3%, *P* < .001 and 101 ± 33 cells/μL, *P* = .06) than in cGVHD patients without IST (21% ± 3%, *P* = .05 and 83 ± 20 cells/μL, *P* = .16), whereas the opposite was observed in RA$^-$ T_{EFF} cells indicating that the latter are IST sensitive. In tolerant patients, the percentage of RA$^-$ T_{EFF} remained marginally increased compared with normal controls (13% ± 2% versus 7% ± 1%, *P* = .05), whereas the percentage of RA$^+$ T_{EFF} was essentially the same as in normal controls (13% ± 2% versus 12% ± 2%, *P* = ns). There was no significant difference in the absolute number of RA$^+$ or RA$^-$ T_{EFF} cells in tolerant

Table 2A. Percentage of CD8$^+$ T Cell Subsets in Chronic GVHD and Tolerant Patients*

	Chronic GVHD Patients				
Percentage of CD8$^+$ T Cells	Without IST (n = 24)†	During IST (n = 20)	Overall (n = 44)	Tolerant Patients (n = 21)	Normal Controls (n = 20)
T_N	**18 ± 3** (<0.001)	**23 ± 4** (0.03)	**20 ± 2** (0.001)	**25 ± 4** (0.05)	35 ± 4
T_{CM}	8 ± 1	5 ± 1	6 ± 1	11 ± 2	8 ± 1
T_{EM}	**15 ± 2** (0.05)	**10 ± 1** (0.001)	**13 ± 1** (0.004)	20 ± 2	19 ± 2
T_{EFF} overall	**40 ± 4** (<0.001)	**38 ± 4** (0.003)	**39 ± 3** (<0.001)	27 ± 4 (0.29)	19 ± 3
RA^- T_{EFF}	**20 ± 2** (<0.001)	10 ± 2	**15 ± 2** (0.006)	**13 ± 2** (0.05)	7 ± 1
RA^+ T_{EFF}	**21 ± 3** (0.05)	**28 ± 3** (<0.001)	**24 ± 2** (0.002)	13 ± 2	12 ± 2
Other‡	19 ± 1	24 ± 2	21 ± 1	17 ± 1	18 ± **2**

GVHD indicates graft-versus-host disease; IST, immunosuppression therapy.
*Data consist of percentage of CD8$^+$ T cells, and values are expressed as mean ± SEM (*P* values). All *P* values represent comparisons between patients and normal controls and are adjusted for age of patients and normal controls. Adjusted *P* values >.05 are omitted.
†The chronic GVHD without IST group consists of 19 patients with "mild" and 5 patients with "moderate/severe" disease. Both subgroups showed similar trends in CD8$^+$ T cell subsets, including an increase in the percentage of T_{EFF} in both "mild" and "moderate/severe" chronic GVHD patients (41% ± 5% and 40% ± 8%), and a decrease in T_N in both mild and moderate/severe GVHD patients (16% ± 3% and 22% ± 7%).
‡The "other" category includes 3 additional CD8$^+$ T cell subsets (CD45RA$^-$CD127$^-$CD62L$^+$, CD45RA$^+$CD127$^-$CD62L$^+$, and CD45RA$^+$CD127$^+$CD62L$^-$).

patients (24.5 ± 5 and 29 ± 8 cells/μL) compared with controls (37 ± 10 and 18 ± 5 cells/μL) (Table 2B and Figure 4B).

In a multivariate regression model, the percentages of T_N and overall T_{EFF} (both RA^- T_{EFF} and RA^+ T_{EFF}), but not T_{CM} or T_{EM}, were associated with patient age. The percentage of T_N was lower in older individuals (−0.7%/year, $P < .001$), whereas the percentage of RA^- and RA^+ T_{EFF} was higher in older individuals (+0.2%/y, $P = .02$; and +0.4%/year, $P < .001$, respectively). The percentage of T_N was lower in CMV seropositive patients (−8.1%, $P = .03$), whereas the percentage of RA^- and RA^+ T_{EFF} was higher in CMV seropositive patients (+7.2%, $P = .01$; and +7.1%, $P = .04$, respectively). The percentage of T_{CM}, but not T_{EM}, was lower in CMV seropositive patients (−5.0%/year, $P = .007$) (Table S2A and Figure 5). The multivariate analysis also confirmed a decrease in absolute T_N counts associated with age (−0.14 cells/μL/year, $P = .005$), and an increase in T_{EFF} cells associated with age (+0.40 cells/μL/year, $P = .04$) (Table S2B). Additionally, the multivariate analysis showed an increase in the percentage T_{EFF} and absolute T_{EFF} cell counts ($P = .005$ and $P = .001$, respectively) and revealed an increase in the absolute T_{EM} cell counts in CMV seropositive patients ($P = .004$). The association between use of IST and a lower percentage of RA^- T_{EFF} in cGVHD patients also remained significant in the multivariate regression analysis ($P = .009$) (Table S2A).

Table 2B. Absolute Number of CD8$^+$ T Cell Subsets in Chronic GVHD and Tolerant Patients*

	Chronic GVHD Patients				
Absolute # CD8$^+$ T Cells	Without IST (n = 22)†	During IST (n = 20)	Overall (n = 42)	Tolerant Patients (n = 19)	Normal Controls (n = 12)
T_N	**37 ± 8** (0.01)	**30 ± 5** (0.005)	**34 ± 5** (0.003)	63 ± 16	84 ± 25
T_{CM}	23 ± 5	14 ± 6	19 ± 4	20 ± 3	21 ± 6
T_{EM}	48 ± 12	39 ± 13	43 ± 9	38 ± 6	46 ± 11
T_{EFF} overall	**171 ± 41** (0.04)	144 ± 50	**158 ± 32** (0.05)	54 ± 12	54 ± 14
RA^- T_{EFF}	**88 ± 23** (0.01)	43 ± 17	**67 ± 15** (0.05)	29 ± 8	18 ± 5
RA^+ T_{EFF}	83 ± 20	**101 ± 33** (0.06)	**92 ± 19** (0.07)	24.5 ± 5	37 ± 10
Other‡	57 ± 8	61 ± 18	59 ± 10	34 ± 5	40 ± 7

GVHD indicates graft-versus-host disease; IST, immunosuppression therapy.
*Data consist of absolute cell counts (cells/μL) expressed as the mean ± SEM (*P* values). All *P* values represent comparisons between patients and normal controls and are adjusted for age of patients and normal controls. Adjusted *P* values >.05 are omitted.
†The chronic GVHD without IST group consists of 18 patients with "mild" and 4 patients with "moderate/severe" disease. Both subgroups showed similar trends in CD8$^+$ T cell subsets, including an increase in the absolute number of T_{EFF} in both mild and moderate/severe GVHD patients (170 ± 48 and 175 ± 71 cells/μL), and a decrease in T_N in both mild and moderate/severe GVHD patients (36 ± 9 and 40 ± 16 cells/μL).
‡The "other" category includes 3 additional CD8$^+$ T cell subsets (CD45RA$^-$CD127$^-$CD62L$^+$, CD45RA$^+$CD127$^-$CD62L$^+$, and CD45RA$^+$CD127$^+$CD62L$^-$).

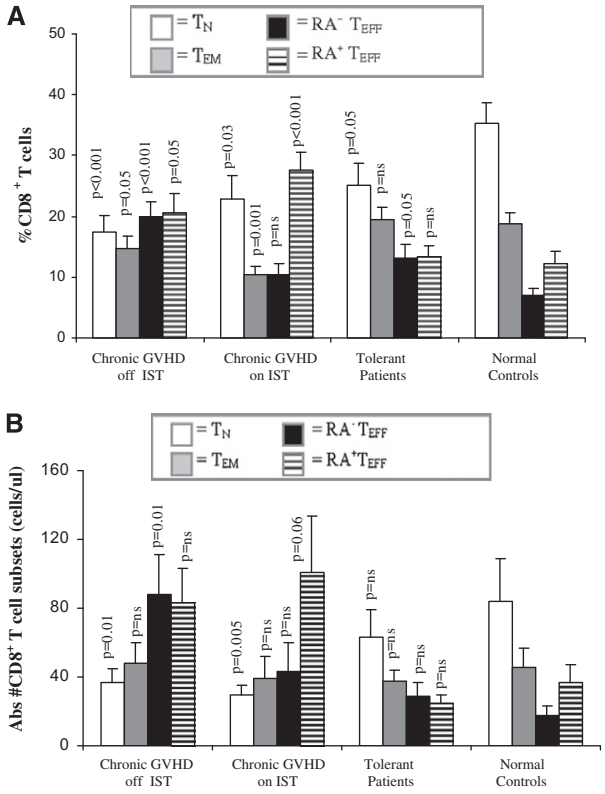

Figure 4. Comparison naïve (T_N), effector memory (T_{EM}), RA^- effector (RA^-T_{EFF}), and RA^+ effector (RA^+T_{EFF}) $CD8^+$ T cells in patients with chronic GVHD, tolerant patients, and normal controls. (A) The data are expressed as percentage of $CD8^+$ T cells. Samples include 24 patients with chronic GVHD off IST (19 mild and 5 moderate/severe disease), 20 patients with chronic GVHD on IST, 21 tolerant patients, and 20 normal healthy controls. (B) The data are expressed as cells per microliter (cells/μL). Samples include 22 patients with chronic GVHD off IST (18 mild and 4 moderate/severe disease), 20 patients with chronic GVHD on IST, 19 tolerant patients, and 12 normal healthy controls. All *P* values represent comparisons between patients and normal controls and are adjusted for age of patients and normal controls.

Activation Markers (HLA-DR, CD95, and PD-1)

The percentage of $HLA\text{-}DR^+$ cells in the $CD8^+$ population was not increased in patients with cGVHD but was increased in tolerant patients compared with controls (9% ± 3% versus 4% ± 1%, $P = .002$) (Table 3). The percentage of $HLA\text{-}DR^+$ cells was lower in older patients (−0.22%/year, $P = .01$), but increased with time from HCT (+1.29%/year, $P < .001$) (Table S3). The percentage of $CD8^+$ cells expressing CD95 (Fas) was significantly increased in patients with cGVHD during IST compared with controls (59% ± 3% versus 40% ± 3%; $P = .005$), and in patients during IST compared with those without IST (59% ± 3% versus 47% ± 4%, $P = .008$) (Tables 3 and S3). The percentage of $CD95^+$ cells was also found to increase with age (+0.5%/year, $P = .005$) (Table S3). CD95 expression was variable among the $CD8^+$ T subsets. In normal controls, CD95 expression was lowest in T_N cells (20% ± 2%), highest in T_{CM} (75% ± 3%), and was higher in RA^- compared with RA^+ T_{EFF} cells (64% ± 4% versus 32% ± 5%, $P < .001$). Similar parallel differences in CD95 expression were observed among T_N, T_{CM}, and RA^+ and RA^- T_{EFF} cells in cGVHD and tolerant patients (data not shown). We found no statistically significant difference in the expression of PD-1, a member of the CD28/CTLA-4 family of T cell regulators, in tolerant patients, or cGVHD patients either during or without IST compared with normal controls. A history of aGVHD, however, was associated with a lower percentage of cells expressing PD-1 (−18.3%, $P = .008$) (Table S3). Among $CD8^+$ T cell subsets, PD-1 expression in patients was lowest in T_{EM} cells and highest in RA^- T_{EFF} cells (28% ± 4% and 45% ± 4%), both similar to normal controls (T_{EM} cells, 37% ± 6%; and RA^- T_{EFF} cells, 52% ± 5%).

Perforin and Granzyme A Expression

We observed a significant increased percentage of $CD8^+$ cells expressing perforin in cGVHD patients overall (46% ± 4%), but not in tolerant patients (29% ± 5%), compared with controls (21% ± 5%) ($P = .003$ and P = ns, respectively) (Table 3). Perforin levels were also higher in CMV seropositive patients (+24.2%, $P < .001$). In contrast, the percentage of $CD8^+$ cells expressing granzyme A was increased in tolerant patients compared with controls (27% ± 5% versus 11% ± 4%, $P = .004$), but not in patients with cGVHD (16% ± 3%, P = ns). Patient age, time from HCT, and CMV serostatus had no significant effect on granzyme expression (Table S3).

Proliferation and Apoptosis

The percentage of $CD8^+$ T cells expressing Ki-67, a marker for recent proliferation, was increased in patients with cGVHD compared with controls (5% ± 1% versus 3% ± 0.5%, $P = .04$), especially in patients who had cGVHD without IST (6% ± 2%, $P = .01$) (Table 3). Similarly, we found a statistically significant increase in the percentage of apoptotic $CD8^+$ T cells in patients with cGVHD compared with normal controls (50% ± 5% versus 31% ± 4%, $P = .01$) (Table 3). The percentages of $Ki\text{-}67^+$ and apoptotic cells in the $CD8^+$ T population of tolerant patients were not significantly different from controls. In cGVHD patients, Ki-67 expression was shown to be significantly increased in $CD8^+$ T_{CM} cells during and without IST ($P = .01$ and $P = .007$, respectively), and in T_{EM} cells in cGVHD patients without IST compared with normal controls (Figure 6). There was also an increased percentage of $Ki\text{-}67^+$ cells in T_N cells in cGVHD patients during IST (2.5% ± 0.5%, $P = .008$), and in tolerant patients (2.0% ± 0.4%, $P = .03$) compared with normal controls (0.4% ± 0.1%). The highest $Ki\text{-}67^+$ expression levels were seen among T_{EFF} cells, especially among RA^- T_{EFF} cells in cGVHD patients without IST.

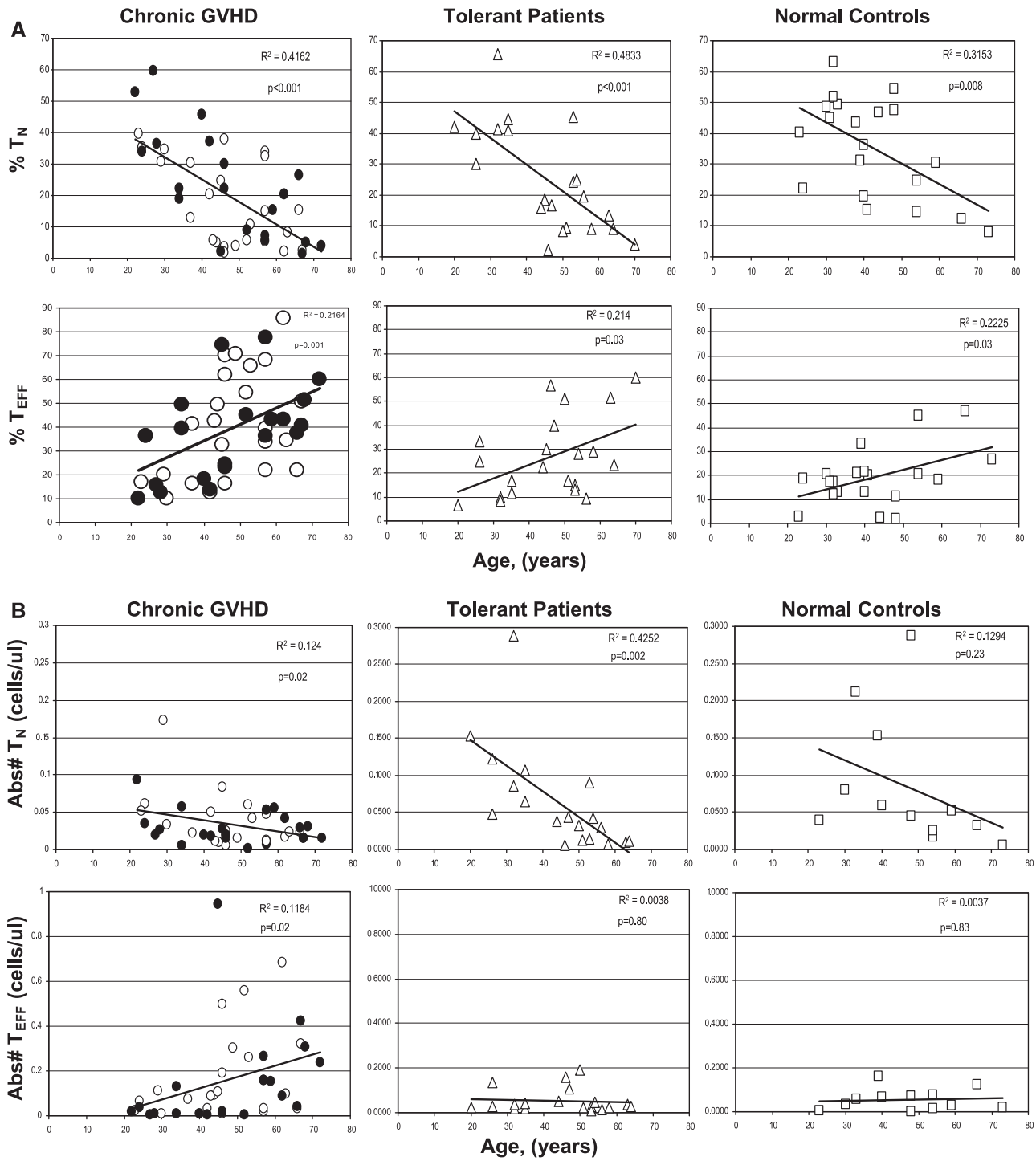

Figure 5. Comparison of the percentage (A) and absolute number (B) of naïve (TN) and effector (TEFF) $CD8^+$ T cells in patients and normal controls according to age. For chronic GVHD patients, open circles represent patients without IST and closed circles represent patients during IST (left panels). Tolerant patients are represented by open triangles (middle panels), and normal controls are represented by open squares (right panels). R^2 value represents the square of the correlation coefficient, and the *P* value is calculated from the R value using GraphPad Software.

During IST, Ki-67 expression was significantly suppressed in RA^+ T_{EFF} cells (6% ± 1% versus 2% ± 0.3%, $P = .02$ and in the RA^- T_{EFF} cells (15% ± 4% versus 5% ± 1%, $P = .05$). These IST-associated effects are consistent with the hypothesis that activation, proliferation, and/or survival of T_{EFF} cells in cGVHD patients is inhibited by the administration of IST.

DISCUSSION

This study population represents a large cross-sectional cohort of patients from 1 year to 23 years (median: 28 months) after HCT who were seen for evaluation and management in our Long-term Follow-Up Clinic. All patients received a systematic

Table 3. Percentage of CD8$^+$ T Cell Activation Markers and Effector Molecules in Chronic GVHD and Tolerant Patients*

	Chronic GVHD Patients				
CD8+ T Cells	Without IST (n = 17)†	During IST (n = 18)	Overall (n = 35)	Tolerant Patients (n = 20)	Normal Controls (n = 18)
%CD95 ‡	47 ± 4	**59 ± 3**§ (0.005)	**53 ± 3** (0.05)	44 ± 3	40 ± 3
%HLA-DR	2 ± 1	3 ± 1	3 ± 0.5	**9 ± 3** (0.002)	4 ± 1
%PD-1	45 ± 6	50 ± 6	48 ± 4	48 ± 5	44 ± 4
%perforin§	**41 ± 5** (0.04)	**51 ± 7** (0.001)	**46 ± 4** (0.003)	29 ± 5	21 ± 5
%granzyme ¶	15 ± 4	16 ± 4	16 ± 3	**27 ± 5** (0.004)	11 ± 4
%Ki-67	**6±2** (0.01)	4 ± 0.4	**5 ± 1** (0.04)	3 ± 0.4	3 ± 0.5
%annexin ⊥	50 ± 8	50 ± 7	**50 ± 5** (0.01)	43 ± 4	31 ± 4

GVHD indicates graft-versus-host disease; IST, immunosuppression therapy.

*Data consist of mean ± SEM and *P* values. All *P* values represent comparisons between patients and normal controls and are adjusted for age of patients and normal controls. Adjusted *P* values >.05 are omitted.

†The chronic GVHD without IST patient group consisted of 12 patients with "mild" and 5 patients with "moderate/severe" disease. There was a non-significant trend for higher perforin expression in patients with "moderate/severe" compared with "mild" disease (52% ± 6% versus 37% ± 7%, *P* = .12), but the expression of Ki-67 was similar in patients with "mild" and "moderate/severe" disease (6% ± 2% and 7% ± 3%).

‡CD95 expression was significantly greater in chronic GVHD patients during IST compared with chronic GVHD patients without IST (59% ± 3% versus 47% ± 4%, *P* = .02).

§Perforin expression in patients was highest in the T_{EFF} subset in both the RA^- and RA^+ T_{EFF} subsets of patients (50% ± 3% and 58% ± 3%), and normal controls (38% ± 6% and 51% ± 7%, respectively).

¶The highest levels of granzyme expression in tolerant patients was observed in T_{EM} cells (43% ± 7%), and in RA^- and RA^+ T_{EFF} cells (37% ± 6% and 41% ± 6%). Among normal controls, granzyme expression in CD8 subsets was also higher in the RA^- T_{EFF}, RA^+ T_{EFF}, and T_{EM} cells (28% ± 7%, 30% ± 8%, and 24% ± 7%) compared with the average for all CD8$^+$ T cells (11% ± 4%).

⊥The study population for the annexin staining included 27 patients (9 with chronic GVHD without IST; 7 with chronic GVHD during IST; and 11 tolerant patients), and 12 normal controls.

and standard assessment for the presence of cGVHD according to the guidelines of the recent NIH Consensus Conference [11]. The patient category "cGVHD without IST" included patients with both mild and moderate/severe disease; however, similar trends in CD8 subsets, activation markers, and effector phenotype were observed in both groups. Patients with no clinical manifestations of cGVHD while still on IST were excluded from the study.

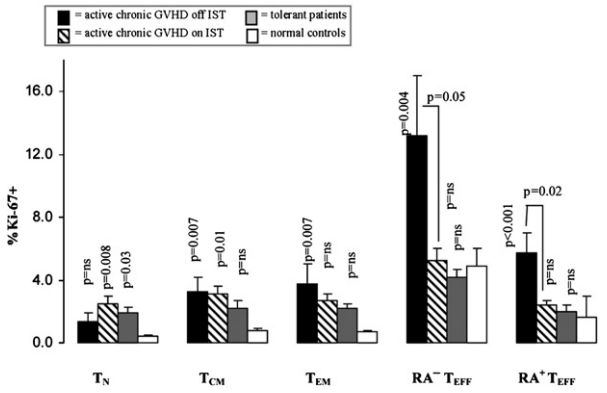

Figure 6. Comparison of percentage of Ki-67 expression in CD8$^+$ naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), RA^- effector (RA^-T_{EFF}), and RA^+ effector (RA^+T_{EFF}) T cells in patients with chronic GVHD, tolerant patients, and normal controls. Samples include 15 patients with chronic GVHD off IST, 14 patients with chronic GVHD on IST, 20 tolerant patients, and 16 normal healthy controls. *P* values represent comparison of patients with normal controls and were calculated using a two-tailed *t* test. All *P* values represent comparisons between patients and normal controls and are adjusted for age of patients and normal controls.

The major finding in this study was an increase in the percentage and absolute number of CD8$^+$ T_{EFF} cells. The increased expression of perforin, Ki-67, and annexin that we observed in patients with cGVHD compared with healthy controls suggests that mature effector T cells are not only expanded in these patients, but that they are also maintained in a state of activation that persists for several months or years after transplantation. Ki-67 expression levels were increased not only in T_{EFF}, but also in T_{CM} and T_{EM}, indicating that there is increased proliferation among these mature CD8$^+$ T cells, a finding that may reflect compensation for the increased recruitment of cells responsive to host alloantigen and subsequent activation-induced cell death. Despite the increased expression of Ki-67 in T_{CM} and T_{EM}, there was no increase in the absolute number of T_{CM} and T_{EM} in cGVHD patients compared with normal controls, suggesting that the levels of T_{CM} and T_{EM} in cGVHD patients, but not the number of T_{EFF} cells, remain under homeostatic control. Two of the markers for T cell activation used in this study, HLA-DR and granzyme, gave unexpected results in tolerant patients. Neither was significantly increased in cGVHD patients, but their expression was increased in tolerant patients, suggesting that the resolution of GVHD can occur without complete normalization of CD8$^+$ T cell phenotype as defined by

homeostasis in normal healthy controls. The functional significance of this observation is unknown, but it is consistent with the hypothesis that clinical tolerance in HCT recipients is induced and maintained by an active cellular mechanism.

In contrast to the expansion of $CD8^+$ T_{EFF} cells in cGVHD patients, there was a reciprocal decrease in the percentages and absolute number of T_N, and an increased expression of Ki-67 by T_N in cGVHD patients overall, consistent with the hypothesis that ongoing alloantigenic stimulation is driving the activation, differentiation, and/or apoptosis of T_N and resulting in the accumulation of T_{EFF} cells. In a multivariate regression model, the percentages of $CD8^+$ T_N and T_{EFF} was found to change significantly with age in both patients and normal controls, with decreasing percentages of T_N and increasing percentages of T_{EFF} in older individuals. However, the absolute number of T_N remained significantly lower in patients with cGVHD even after adjusting for the age effect. This may be because of limited thymic function after HCT, increased activation induced cell death, a deficiency of growth factors, or other changes in the lymphoid microenvironment that compromise the production and survival of T cells. These changes may be further compounded by cytolytic and inflammatory consequences of cGVHD.

The use of IST had no apparent effect on the total $CD3^+$ and $CD8^+$ T cell counts in cGVHD patients, whereas $CD4^+$ T cell counts were significantly decreased in cGVHD patients during IST, suggesting that $CD4^+$ T cells are more sensitive to IST than $CD8^+$ T cells in these patients. IST was also associated with a decrease in the percentage and absolute number of RA^- T_{EFF} and an increase in the percentage and absolute number of RA^+ T_{EFF}, suggesting that RA^- T_{EFF} are relatively sensitive to IST, whereas RA^+ T_{EFF} are relatively resistant. This effect may be explained partly by a decrease in apoptosis and Fas expression in the RA^+ T_{EFF} subset, compared with the RA^- T_{EFF} subset and the overall $CD8^+$ T cell population (data not shown). The trend toward normalization of the percentage of T_N in tolerant patients is consistent with the clinical observation that immune reconstitution after HCT is delayed in the presence of cGVHD.

CMV serostatus of the patient had a significant effect on CD8 T cell subsets in this cohort of HCT patients, consistent with what is known about the alterations in peripheral T cell homeostasis associated with latent CMV infection [15]. CMV seropositive patients in our HCT cohort had higher $CD3^+$ and $CD8^+$ T cell counts overall, but a lower percentage of T_N, and a higher percentage and absolute number of $CD8^+$ T_{EFF} cells, compared with seronegative patients. Peggs et al. studied T cell reconstitution during the first 6 months following autologous HCT recipients and observed increased $CD8^+$ T cell counts in patients with active CMV infection compared with patients who remained free of CMV infection [16]. The CMV effect on $CD8^+$ T_N and T_{EFF} in our study paralleled the independent age effect. In a separate study, we found no statistically significant association of latent CMV infection with risk of cGVHD (Flowers et al., unpublished results).

The first differentiation markers shown to be helpful in defining T cell subsets were CD45RA and CCR7 in a study by Sallusto et al. [18], where it was demonstrated that "central memory" and "effector memory" $CD8^+$ T cells could be distinguished by the expression of $CD45RA^-CCR7^+$ and $CD45RA^-CCR7^-$, respectively. We chose an alternative approach to defining mature central memory and effector cells based on studies by Bachmann et al. [19] and Huster et al. [20]. Huster et al. [20] demonstrated that CD127 is a marker for memory $CD8^+$ T cells in the mouse, and they showed that $CD127^{high}CD8^+$ T cells had a high level of proliferation and were capable of long-term survival compared with $CD127^{low}$ $CD8^+$ T cells. They also demonstrated that CD62L and CD127 expression following antigenic priming distinguish T_{CM} ($CD127^{high}CD62L^{high}$) from T_{EM} ($CD127^{high}CD62L^{low}$) $CD8^+$ T cells. Bachmann et al. examined changes in $CD62L^+CD127^+$ T_{CM}, $CD62L^-CD127^+$ T_{EM}, and $CD62L^-CD127^-$ T_{EFF} $CD8^+$ T cells after acute and chronic viral infection in mice. They showed that $CD62L^-CD127^+$ T_{EM} expand early after antigenic stimulation, and that additional stimulation leads to down-regulation of CD127 expression and a higher proportion of $CD62L^-CD127^-T_{EFF}$, whereas cessation of stimulation leads to reexpression of CD62L and increase in the percentage of $CD62L^+CD127^+$ T_{CM} [19]. They also showed that T_{EFF} display high levels of lytic activity and cytokine production after antigenic stimulation. T_{CM}, however, showed high levels of IL-2 secretion and proliferation [19]. In HIV patients with advanced disease and increased viral load, Pairidini et al. [21] demonstrated that decreased CD127 expression by $CD8^+CD62L^-$ cells corresponded to an activated phenotype with increased production of interferon-gamma (IFN-γ) and expression of Ki-67. These findings parallel our observations of an overall decrease in CD127 expression and increase in $CD62L^-CD127^-$ T_{EFF} associated with cGVHD, whereas normalization of CD127 expression and percentage of T_{EFF} correlates with disease resolution and the development of tolerance.

There has been little information available about $CD8^+$ T cell subsets in cGVHD. Yamashita et al. [9] measured $CD8^+$ T_{CM} as $CD45RA^-CCR7^+$ and reported a roughly 2-fold increase in the percentage of $CD8^+$ T_{CM} in cGVHD patients compared with normal controls (23.3% ± 2.0% versus 11.8% ± 1.0%, $P < .0001$). We defined T_{CM} as explained above ($CD45RA^-CD62L^+CD127^+$) and observed no significant difference in either the percentage or absolute

number of T_{CM} in cGVHD patients compared with normal controls, with only a nonsignificant trend for lower numbers of T_{CM} during IST. D'Asaro et al. [10] showed an increased in the percentage of $CD45RA^+CCR7^-$ cells, which they termed T_{EMRA} cells, and a decrease in percentage of $CD8^+$ T_N cells in a study of 12 patients with cGVHD, all of which were on IST at the time of study. We also observed an increase in a $CD45RA^+$ T_{EFF} subset in patients with cGVHD overall, but this increase was only marginal in patients without IST compared with controls (21% ± 3% versus 12% ± 2%, $P = .05$), whereas the increase was much more significant in cGVHD patients during IST (28% ± 3.0%, $P < .001$), suggesting that use of IST leads to an expansion rather than a reduction of this RA^+ T_{EFF} subset. The RA^- T_{EFF} population, however, is shown to be more significantly increased in patients with GVHD without IST and, in contrast to the RA^+ T_{EFF} population, is decreased to normal levels in patients during IST. $CD45RA^+$ T_{EFF} have been described as cells approaching "end-stage differentiation" by some investigators, including Almanzar et al. [22] in a study of CMV immunity. $CD45RA^+$ T_{EFF} are not a uniform population but variably express granzyme B, tumor necrosis factor-alpha (TNF-α), IFN-γ, and FasL [23]. Carrasco et al. [24] studied clonal human effector T cell responses ex vivo in a melanoma model and demonstrated that $CD45RA^+CCR7^-$ MAGE-3 specific cytotoxic T cells (CTL) could proliferate after rechallenge with antigen. These cells subsequently lose expression of CD45RA and temporarily express CCR7. Expression of CD45RA recovered, and CCR7 expression was lost again, at 10 to 15 days following antigen stimulation. These investigators concluded that CD45RA expression is not a marker of terminal differentiation in memory $CD8^+$ T cells, but rather that $CD45RA^+CCR7^-CD8^+$ T cells are "transitional" or "stable resting" effector T cells. Assuming that CD45RA expression by $CD62L^-CD127^-CD8^+$ T cells in cGVHD patients is analogous to the ex vivo tumor model of Carrasco et al. [24], we would infer that RA^- T_{EFF} have recently encountered and responded to host alloantigen, whereas RA^+ T_{EFF} have not. The concept that RA^- T_{EFF} are activated while RA^+ T_{EFF} are quiescent is consistent with the observation that the percentage of RA^- T_{EFF} is reduced in cGVHD patients during IST indicating the effectiveness of IST in blocking activation of $CD8^+$ T_{EFF}, whereas the relative number of quiescent RA^+ T_{EFF} is increased in patients receiving IST.

Clonal deletion and T cell exhaustion have previously been implicated in the development of tolerance. Programmed Death 1 (PD-1) is a cell-surface receptor that has been shown to be involved in the negative regulation of $CD8^+$ T cells and has been implicated in the development of T cell anergy or nonresponsiveness in animal models of chronic infection. Several recent clinical studies have shown an up-regulation of PD-1 associated with T cell exhaustion of $CD8^+$ T cells in patients chronically infected with HIV and HCV [25-28]. We observed no significant difference in PD-1 expression in patients with cGVHD or in tolerant patients compared with healthy controls, suggesting that PD-1 probably does not mediate the development of nonresponsiveness to host alloantigen in HCT recipients. Evidence for clonal deletion by activation induced cell death, however, is supported by the evidence showing increased expression of Ki-67 and increased apoptosis in patients with cGVHD compared with tolerant patients and normal controls.

In summary, the results reported here support the hypothesis that there is a sustained activation and expansion of $CD8^+$ T cells in patients with cGVHD. The relevant findings include an overall decrease in CD127 expression, an expansion in the percentage and absolute number of $CD8^+$ T_{EFF} cells, and an increase in perforin, Ki-67, and annexin expression in patients with cGVHD. The increase in effector T cells was most prominent in older patients and patients who were CMV seropositive before transplantation. The increase in T_{EFF} cells was accompanied by a reciprocal decrease in T_N, whereas the absolute number of T_{CM} and T_{EM} remained within the normal range both in cGVHD and tolerant patients. Perforin, Ki-67, and annexin levels in $CD8^+$ T cells normalize in tolerant patients, but HLA-DR and granzyme A expression are increased in tolerant patients. PD-1 expression was within the normal range in both cGVHD and tolerant patients. IST use was shown to result in a decrease in the relative and absolute number of $CD8^+$ T cells that were RA^- T_{EFF}, and an increase in RA^+ T_{EFF} cells. Overall, these results are consistent with the hypothesis that $CD8^+$ T cells play a role in mediating the immunopathology of cGVHD, and they furthermore suggest that tolerance after HCT does not necessarily correlate with a normalization of the $CD8^+$ T cell phenotype. Although our study was limited to a cross-sectional analysis of cGVHD and tolerant patients, the evidence provided are sufficiently compelling to prompt future longitudinal studies that more thoroughly explore differences in signaling, intracellular pathways, and effector functions of $CD8^+$ T cells in these patients.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbmt.2011.03.007.

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